

Remarks

Claims 1-3 and 6-15 were pending prior to this Response, with claims 8-12, 14 and 15 having been withdrawn from further consideration. By the present communication, no claims have been added or canceled, and claims 1-3 and 7 have been amended to define Applicants' invention with greater particularity. The amendments do not raise any issues of new matter and the amended claims do not present new issues requiring further consideration or search. Support for amended claim 2 may be found, among others, at page 10, lines 1-3. Accordingly, claims 1-3 and 7 are currently pending in this application.

Claim Objections

Applicants respectfully traverse the objection to claim 2 as allegedly failing to further limit the subject matter of a previous claim. Applicants have amended claim 2 to set forth the retention of serine, glutamic acid and lysine residues. Accordingly, Applicants respectfully request withdrawal of the objection.

Rejection under 35 U.S.C. § 112, Second Paragraph

Applicants respectfully traverse the rejection of claims 1-3 and 7 as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner alleges that in claim 1 it is unclear what is intended by the recitation, "...except that and lysine residues of the natural toxin in its amino acid sequence, except that a formalin...." Applicants have amended claim 1 to correct the apparent typographical error by removing the first "except that" from the recitation. Withdrawal of the rejection is respectfully requested.

Further, the Examiner alleges that claims 2 and 3 depend on claim 1, but claim 1 refers to two different toxins. In order to reduce the issues and further prosecution, Applicants have amended claims 2 and 3 to clarify which toxin is being further limited. Withdrawal of the rejection is respectfully requested.

Finally, the Examiner alleges that claim 7 recites a dependency to a canceled claim. In

order to reduce the issues and further prosecution, Applicants have amended claim 7 to correct the claim dependency. Withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. § 102

Applicants respectfully traverse the rejection of claims 1, 3, 6, 7 and 13 under 35 U.S.C. §102(b) as allegedly anticipated by Germanier, et al (hereinafter "Germanier"). Specifically, the Examiner alleges that Germanier discloses a purified and attenuated cholera toxin, wherein the toxin has a residual toxic activity of less than 1/2000 that of the natural toxin. Applicants submit that Germanier discloses cholera toxin (CT) as a starting material, which is first converted into procholeraenoid by heat treatment, and then the procholeraenoid is treated with formalin to obtain an attenuated toxin. In contrast, the attenuated toxin of the present invention is produced *without* the heat treatment step. In other words, the claimed attenuated toxin is produced by directly treating CT with formalin, rather than treating procholeraenoid with formalin.

Applicants further submit that the difference in production methods results in structural differences of the resulting attenuated toxins. The structural differences between CT and procholeraenoid are shown in the table below. CT contains many cysteine residues, which form stable disulfide bonds (SS bonds) in a native environment (Lai, CY *et al.*, Biochem Biophys Res Commun. 116:341-8, 1983) (Abstract attached as Exhibit 1). When CT enters the body, the -SH group of the cysteine residues is released, thereby allowing the cysteine residues to form new SS bonds, which exerts toxin activity. Due to the SS bonds within the molecule, heat-treated CT undergoes structural and functional changes, resulting in procholeraenoid, which has entirely different characteristics. Attached as Exhibit 2, Pierce, *et al.* describe "procholeraenoid" as a stable, high molecular weight aggregate containing both the A and B subunits (see p. 1112, right column, Pierce, *et al.*, Infection and Immunity, 40(3):1112-8, 1983). Since the structural differences between CT and procholeraenoid is evident, Applicants submit that one of skill in the art would unambiguously distinguish the adjuvant of the present invention (comprising an attenuated CT) from the attenuated procholeraenoid of Germanier.

	Untreated CT	Heat-treated CT (i.e., procholeraeagenoid)
Solubility in water	Soluble	Insoluble (syrup or rubber-like form)
Protein Denaturation	Not denatured	Denatured
Molecular weight	Constant at 85 kD	Multimeric complex at 85 kD or greater
Amino Acid Sequence (primary structure)	Constant	Not constant (may have many changes in primary structure)
SS Bond in the Amino Acid Sequence	Positions and the number of SS bonds are constant)six bonds between the A1 and A2 subunits and within the B subunit)	Positions of SS bonds are not constant or not clear
Protein Tertiary Structure	Constant	Not constant
Toxin Activity	Active	Less active than the untreated CT

Further, Applicants submit that Germanier is absolutely silent with regard to the adjuvant activity of the attenuated procholeraeagenoid. Germanier discloses that the procholeraeagenoid obtained by heat and formalin treatment is "antigenic" (see page 1696). However, Germanier does not disclose that the procholeraeagenoid retains an activity to enhance production of an antibody specific to an antigen other than the procholeraeagenoid itself (i.e., adjuvant activity). The only disclosure upon which Germanier may rely that relates to antibody production against antigens other than procholeraeagenoid is, "After immunizing rabbits with 100 µg of formalinized procholeraeagenoid, a weak but distinct rise in vibriocidal antibodies was observed." (p. 1696, right column, first full paragraph). However, in the same paragraph, Germanier concludes that residual somatic antigens present in the toxin preparation S-2, which was used to produce the procholeraeagenoid, would be the cause of the vibriocidal antibody production. Germanier suggests using the purer toxin preparation A-2 to prepare a toxoid free of somatic antigens (p. 1697, last paragraph). Accordingly, one of skill in the art may, at most, recognize that (i) some somatic antigens inactivated neither by heat nor by formalin treatment may exist in Germanier's purified toxin preparation, and (ii) to eliminate such contaminating somatic antigens, a toxin preparation that has been highly purified through gel filtration or such techniques should be used

to produce an attenuated procholera toxin. However, Applicants submit that one of skill in the art cannot reach the conclusion that Germanier's attenuated procholera toxin retains an adjuvant activity.

Anticipation under 35 U.S.C. § 102(b) requires that the reference recite each and every element of the claims in a single document. Since Germanier fails to disclose each and every element of the invention adjuvant, as defined by amended claim 1, Applicants respectfully submit that the Examiner has failed to establish anticipation under 35 U.S.C. § 102 (b) over Germanier, et al. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

Rejection under 35 U.S.C. § 103

Applicants respectfully traverse the rejection of claim 7 under 35 U.S.C. § 103(a) as allegedly unpatentable over Germanier. The burden of proof in establishing a *prima facie* case of obviousness under § 103 clearly rests with the Patent Office. *In re Piasecki*, 745 F.2d 1468, 1472 (Fed. Cir. 1984). In establishing a *prima facie* case, the Patent Office, among other things, must show that (1) the prior art would have suggested to those of ordinary skill in the art that they should make the claimed invention and (2) that the prior art would have revealed a reasonable expectation of success. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991). "Both the suggestion and the reasonable expectation of success must be found in the prior art, not in the applicant's disclosure." *Id.* Thus, "particular findings must be made as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed." *In re Kotzab*, 217 F.3d 1365, 1371 (Fed. Cir. 2000). Further, when relying on the knowledge of persons of ordinary skill in the art, the Patent Office must "explain what specific understanding or technological principle within the knowledge of one of ordinary skill in the art would have suggested the combination." *In re Rouffet*, 149 F.3d 1350, 1357 (Fed. Cir. 1998). "The factual inquiry whether to combine references must be thorough and searching. It must be based on objective evidence of record. This precedent has been reinforced in myriad decisions, and cannot be dispensed with." *In re Sang Su Lee*, 277 F.3d

1338, 1343 (Fed. Cir. 2002) (citations omitted).

Specifically, the Examiner alleges that it would have been obvious to one of skill in the art to remove all toxicity from the toxin to ensure its safe use with whole cell vaccines against *Vibrio cholerae* infection. Applicants cannot find disclosure of Germanier for “the administration of the detoxified toxin with whole cell vaccines against *Vibrio cholerae* infection.” As such, Applicants invite the Examiner to identify the specific passage within Germanier that discloses administration against *Vibrio cholerae* infection.

As discussed above, the structure of the claimed attenuated CT is different from that of the attenuated procholeraenoid of Germanier. The present invention teaches that a high degree of detoxification can be accomplished by simply formalin-treating a toxin that retains serine residues, glutamic acid residues, and lysine residues of the natural toxin. This detoxification is accomplished without treatment with heat. Further, Germanier is absolutely silent with regard to the retention of an adjuvant activity by the resulting procholeraenoid.

Accordingly, Applicants submit that Germanier does not suggest that use of CT, rather than procholeraenoid, would result in an attenuated toxin. Withdrawal of the rejection is respectfully requested.

In re Application of:
Aizawa et al.
Serial No.: 09/830,019
Filed: September 21, 2001
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PATENT
Attorney Docket No. SHIM1120


Conclusion

In summary, for the reasons set forth herein, Applicants maintain that claims 1-3 and 7 clearly and patentably define the invention and respectfully request that the Examiner withdraw all rejections and pass the application to allowance. If the Examiner would like to discuss any of the issues raised in the Office Action, the Examiner is encouraged to call the undersigned so that a prompt disposition of this application can be achieved.

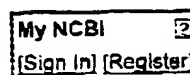
A check in the amount of \$120.00 is enclosed as payment for the one-month Petition for Extension of Time fee. No other fee is believed due in connection with the filing of this Response. However, if any fee is due, the Commissioner is hereby authorized to charge any additional amounts required by this filing, or credit any overpayment, to Deposit Account No. 07-1896 referencing the above-identified attorney docket number. A duplicate copy of the Transmittal Sheet is enclosed.

Respectfully submitted,

Date: August 16, 2006


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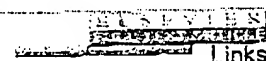
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☐ 1: Biochem Biophys Res Commun. 1983 Oct 14;116(1):341-8.**Location and amino acid sequence around the
ADP-ribosylation site in the cholera toxin active subunit A1.****Lai CY, Xia QC, Salotra PT.**

Renatured, S-carboxymethylated subunit A1 of cholera toxin possess the ADP-ribose transferase activity (Lai, et.al., Biochem. Biophys. Res. Commun. 1981, 102, 1021). In the absence of acceptor self ADP-ribosylation of A1 subunit was observed. Stoichiometric incorporation of ADP-ribose moiety was achieved in 20 min at room temperature in a 0.1 - 0.2M PO₄(Na) buffer, pH 6.6. On incubation of the complex with polyarginine, 75% of the enzyme-bound ADP-ribose moiety was transferred to the acceptor in 25 min. The ADP-ribosylated A1 was stable at low pH, and on cleavage with BrCN, the ADP-ribose moiety was found associated with peptide Cn I, the COOH-terminal fragment of A1 subunit. On further fragmentation with cathepsin D, a dodecapeptide containing ADP-ribose moiety was isolated whose structure was determined as:

Asp-Glu-Glu-Leu-His-Arg-Gly-Tyr-Arg*-Asp-Arg-Tyr. The Arg* in the peptide was indicated to be the site of ADP-ribosylation.

PMID: 6315008 [PubMed - indexed for MEDLINE]

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Procholeragenoid: A Safe and Effective Antigen for Oral Immunization Against Experimental Cholera

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The immunogenicity and safety of procholeragenoid, a minimally toxic, heat-induced aggregate of cholera toxin (CT), were studied in enterically immunized rats and dogs. Although 99% less toxic than CT, procholeragenoid was only slightly less efficient in causing jejunal anti-CT responses in rats; in contrast, choleragenoid, the nontoxic B subunit pentamer of CT, was much less effective. The immunogenicity of procholeragenoid was due almost entirely to its large-molecular-weight components ($MW = 10^6$ to 10^7) and was markedly reduced by preincubation with GM_1 ganglioside or treatment with Formalin to eliminate residual toxicity. These findings suggest that molecular aggregation, binding to GM_1 receptors on cell membranes, and stimulation of cellular adenylate cyclase each contributed to the effectiveness of procholeragenoid as a mucosal immunogen. In dogs, oral immunization with five 500- μ g doses of procholeragenoid evoked vigorous anti-CT responses in jejunal mucosa without causing significant diarrhea. When subsequently challenged with virulent *Vibrio cholerae*, immunized dogs showed 83% protection against the development of severe or lethal diarrhea compared with non-immunized controls. These results confirm a protective role for mucosal antitoxin in experimental cholera and show that procholeragenoid is both safe and effective as an oral immunogen. Procholeragenoid, combined with other antigens of *V. cholerae*, may constitute a simple, safe, and effective oral vaccine for cholera.

Recent studies have shown that antibodies against cholera toxin (CT) and *Vibrio cholerae* lipopolysaccharide act synergistically to protect experimental animals challenged with living virulent *V. cholerae* organisms (10, 14, 22). In some of these studies, nonliving antigens were given entirely by mouth, and the observed protection was probably due to secretory immunoglobulin A antibodies elaborated in the intestinal lamina propria (14). These findings suggested that a multiantigen oral vaccine which could evoke secretory anti-CT and anti-lipopolysaccharide and perhaps secretory antibodies to other antigens of *V. cholerae*, would be much more effective than a single purified antigen and would find practical use in humans.

However, a safe and efficient method for evoking a protective mucosal anti-CT response has not been established, although CT and several nontoxic derivatives of CT have been studied as oral immunogens. CT given orally is an extremely effective mucosal immunogen (4, 12), but minimum immunizing doses also cause tran-

sient diarrhea, at least in some animals (14); whether oral CT can immunize humans without causing diarrhea has not been determined. Choleragenoid, the B subunit pentamer of CT, does not cause diarrhea, but it is poorly immunogenic when given orally or intrainestinally to animals that have not been primed enterically with CT (12, 14). Similarly, CT which has been detoxified by treatment with Formalin or glutaraldehyde is safe, but poorly immunogenic, when given by mouth to nonimmune animals or humans (7, 17, 18).

Procholeragenoid is an antigenic derivative of CT, containing both the A and B subunits, that has not been extensively studied as an oral immunogen. Finkelstein et al. first showed that heat treatment causes CT to form a stable high-molecular-weight aggregate. They termed this material "procholeragenoid" and showed that it was at least 95% less toxic than CT (2). In further studies, Fujita and Finkelstein showed that mice fed procholeragenoid were significantly protected against subsequent challenge of a

ligated intestinal loop with living *V. cholerae* (5), and similar results were obtained in rabbits by Peterson (11).

Because procholeraenoid is antigenic, but only minimally toxic, it seemed appropriate to conduct further studies of its effectiveness and safety as a mucosal immunogen. We have compared the abilities of CT, procholeraenoid, and choleraenoid to evoke mucosal anti-CT responses in rats, and we have studied procholeraenoid as an oral vaccine against experimental cholera in dogs. Our results, presented here, show that enterically administered procholeraenoid is only slightly less effective than CT in causing mucosal anti-CT responses in rats, evokes vigorous mucosal anti-CT responses in dogs without causing diarrhea, and significantly protects dogs against challenge with living *V. cholerae* organisms.

MATERIALS AND METHODS

Animals. Rats were inbred females of the Lewis strain (LEW/CrI BR) obtained from Charles River Breeding Laboratories, Wilmington, Mass. Housing was in a conventional rodent colony. Rats weighed 125 to 150 g (7 to 8 weeks old) when first studied. Dogs were healthy mongrels of either sex weighing 9 to 20 kg when challenged. Before immunization, they were quarantined for 2 weeks, dewormed, and immunized for rabies and distemper.

Antigens. All antigens were provided in lyophile. Purified CT was NIH lot 0972, prepared by Richard Finkelstein and supplied by Robert Edelman, National Institute of Allergy and Infectious Diseases. Choleraenoid was isolated from purified CT as described previously (3) and was provided by Richard Finkelstein. Procholeraenoid was prepared by heating purified CT, derived from *V. cholerae* Inaba 569B, in Tris-EDTA buffer for 20 min at 60°C as previously described (6). For some studies, procholeraenoid was treated with 0.2% Formalin for 60 h at 30°C to virtually eliminate residual toxicity (6).

Chromatography of procholeraenoid was performed by using a Sepharose Cl 4B column (2.5 by 65 cm). Procholeraenoid (60 mg in 10 ml of Tris-EDTA buffer) was applied to a column pre-equilibrated with Tris-EDTA buffer. The column was eluted at a flow rate of 45 ml/h. Fractions of 4.5 ml were collected, and their optical density at 280 nm was measured (Fig. 1). Fractions were pooled to represent peaks I, II, and III as shown. Peak II appeared to be a shoulder of peak I, and its separation from peak I was arbitrary; peak III, however, was distinct from peaks I and II. Peaks I and II had average molecular weights of approximately 10^7 and 10^6 , respectively. These were concentrated about 10-fold by ultrafiltration on Amicon PM 30 filter membranes (Amicon N.V., Netherlands); the concentrated solutions were then mixed with an equal volume of 10% lactose, for stabilization, and lyophilized. Peak III had an average molecular weight of 56,000, which corresponds to choleraenoid. It was similarly concentrated by using an Amicon UM-10 filter membrane and was lyophilized without the addition of lactose.

Immunization. Antigens were reconstituted and diluted in 0.05 M borate-buffered saline or phosphate-

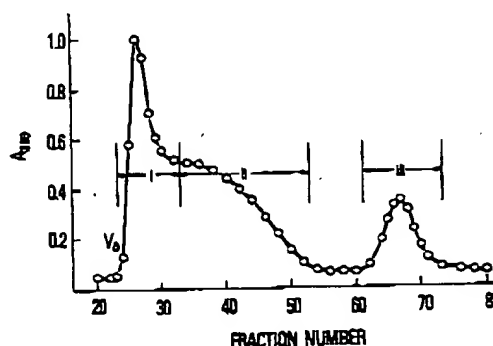


FIG. 1. Chromatography of procholeraenoid on Sepharose 4B. Procholeraenoid (60 mg) was applied to a Sepharose 4B Cl column (2.5 by 65 cm) and eluted with Tris-EDTA buffer at a flow rate of 45 ml/h. Fractions of 4.5 ml were collected, their optical density at 280 nm was measured, and they were pooled to form peaks I, II, and III as shown.

buffered saline, each containing 0.02% gelatin at pH 7.4. In some instances, antigens were preincubated for 30 min at room temperature with purified GM₁ ganglioside (Supelco, Inc., Bellefonte, Pa.), 60 µg/ml, before being administered.

Rats were immunized by direct intraduodenal injection of antigen in a volume of 0.5 ml, using a small laparotomy as described previously (16); most studies involved two antigen doses given with a 14-day interval. Dogs were immunized by giving them antigen through an orogastric tube, also as described elsewhere (18). In brief, individually caged dogs were fasted overnight and then were given 50 ml of 6% NaHCO₃, followed immediately by 100 ml of 2% Casamino Acids (Difco Laboratories, Detroit, Mich.) containing 500 µg of procholeraenoid; feeding was resumed after 5 h, and dogs were observed for 24 h for diarrhea.

Assays for CT activity. Residual CT activity in procholeraenoid was measured by the rabbit skin capillary permeability assay, as described elsewhere (1). Results are expressed in 4-mm bluing doses (BD₄) per milligram of injected material. One BD₄ is that amount of CT or procholeraenoid which causes a blue lesion with a mean diameter of 4 mm 22 to 24 h after intracutaneous injection (1).

Antitoxin responses. Antitoxin-containing plasma cells (ACC) were enumerated in jejunal biopsies from rats or dogs, using a previously described fluorescent-antibody technique (16). In rats, biopsies were taken 5 days after the second injection of antigen; in dogs, they were obtained 6 to 7 days after the final antigen dose. These intervals were chosen to detect ACC when they were most numerous (16). Results are expressed as ACC per cubic millimeter in the crypt region of jejunal lamina propria. ACC were never seen in biopsies from nonimmunized animals (15, 16). ACC responses are expressed as geometric means because these reflect the logarithmic manner in which the ACC response expands after immunization and because mucosal protection against challenge with CT correlates linearly with the geometric mean frequency of ACC in the lamina propria (15).

Antitoxin was assayed in serum by using a previously described mouse adrenal tumor cell assay (20). Antitoxin titers were determined by comparing each sample with a simultaneously titrated standard serum containing 4,470 antitoxin units per ml [lot EC3 (A-67)-B, manufactured by the Swiss Serum and Vaccine Institute, Berne, Switzerland]. Preimmunization titers in dogs were consistently less than 1 U/ml, which was the sensitivity of the assay.

Challenge of dogs with virulent *V. cholerae* organisms. Preparation of the challenge inoculum and the challenge technique were as described elsewhere (17). Fasting dogs were inoculated with 1.0×10^{11} to 2.1×10^{11} viable *V. cholerae* Ogawa 395 organisms by orogastric tube. At each challenge, immunized dogs and an equal number of nonimmunized controls received identical inocula. The variation in the number of viable bacteria in the inocula was within a range that does not affect the attack rate for diarrhea in nonimmunized dogs (21).

Dogs were observed in metabolic cages for 5 days after challenge. Food and water were withheld for the first 18 h so that liquid stool output could be accurately measured. Results of challenge were classified as (i) no diarrhea, (ii) mild diarrhea (one or more watery stools, but no weakness, lethargy, or decrease in skin turgor), (iii) severe diarrhea (voluminous watery diarrhea, decreased skin turgor, and weakness or lethargy), and (iv) lethal diarrhea. Diarrhea usually began less than 18, and often less than 8, h after challenge; about 70% of deaths occurred within the first 24 h. Challenge outcome was also expressed as the output of liquid stool (in milliliters per kilogram of body weight) during the first 18 h after challenge, when stool volume was usually the greatest (14).

Protection of immunized dogs was analyzed by the chi-square test, comparing the number of immunized and control dogs in three outcome categories: no diarrhea, mild diarrhea, and severe or lethal diarrhea.

RESULTS

Toxicity of procholeraenoid preparations. The toxicities of various procholeraenoid preparations and fractions, as measured by the skin capillary permeability assay, are summarized in Table 1. Procholeraenoid and its three separated peaks had residual toxicities ranging from 0.4 to 1.4% of that of purified CT. Treatment of procholeraenoid with Formalin reduced its toxicity about 1,000-fold.

TABLE 1. Relative toxicity of procholeraenoid preparations

Material	BD ₅₀ /mg ($\times 10^5$)	% Toxicity relative to CT
Cholera toxin	900	
Procholeraenoid	4.0	0.4
Peak I	4.2	0.5
Peak II	13.0	1.4
Peak III	5.7	0.6
Formalin-treated procholeraenoid	0.005	0.0006

Immunogenicity of CT, procholeraenoid preparations, and choleraenoid in rats. The relative efficacies of CT, procholeraenoid, and Formalin-treated procholeraenoid as primary enteric immunogens were studied in rats. The first studies involved intraduodenal priming with various doses of one of the antigens, followed 14 days later by a 12.5- μ g intraduodenal booster dose of CT. The resultant secondary ACC responses are summarized in Fig. 2. Peak responses achieved by CT and procholeraenoid were similar, but the dose of procholeraenoid required to prime for an intermediate booster response of 1,000 ACC/mm³ was slightly larger: 1.5 times that of CT. In contrast, Formalin-treated procholeraenoid was much less effective; at the highest dose tested (50 μ g), it primed for a secondary ACC response that was only 10% of that seen in rats optimally primed with 3.2 μ g of CT.

In further studies, rats were primed and challenged intraduodenally with identical, graded doses of a single antigen: CT, procholeraenoid, or choleraenoid. The order of immunizing efficiency of these materials was CT > procholeraenoid > choleraenoid (Fig. 3). Moreover, peak responses achieved by repeated immunization with choleraenoid were only 10% as great as those in CT-immunized rats. In contrast, rats given 12.5- or 50- μ g doses of procholeraenoid developed ACC responses similar to those evoked by the same doses of CT.

The immunogenicity of each of the three chromatographically separated peaks of procholeraenoid is compared with that of whole procholeraenoid and CT in Table 2. In general, peaks I and II were similar to whole procholeraenoid with respect to priming for an ACC response, peak II being slightly more effective than peak I. In contrast, peak III was virtually ineffective for enteric priming. Table 2 also shows the effect of preincubation of these materials with GM₁ ganglioside. In every instance, priming efficiency was markedly diminished by preexposure to ganglioside.

Safety and efficacy of procholeraenoid as an oral immunogen in dogs. Dogs were immunized orally with 500- μ g doses of procholeraenoid on days 0, 21, 42, 49, and 56. This regimen was based on evidence that maximum ACC responses and protection occurred in dogs given multiple oral doses of purified CT (14). Jejunal biopsies to assess ACC responses were obtained from some dogs 6 to 7 days after the final dose of antigen. Challenge with viable *V. cholerae* Ogawa 395 organisms was 21 days after the final immunization.

Mild diarrhea occurred in 6% of dogs given the first, second, or third oral dose of procholeraenoid (Table 3). This figure does not differ significantly from the previously described 4%

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ORAL IMMUNIZATION WITH PROCHOLERAGENOID 1115

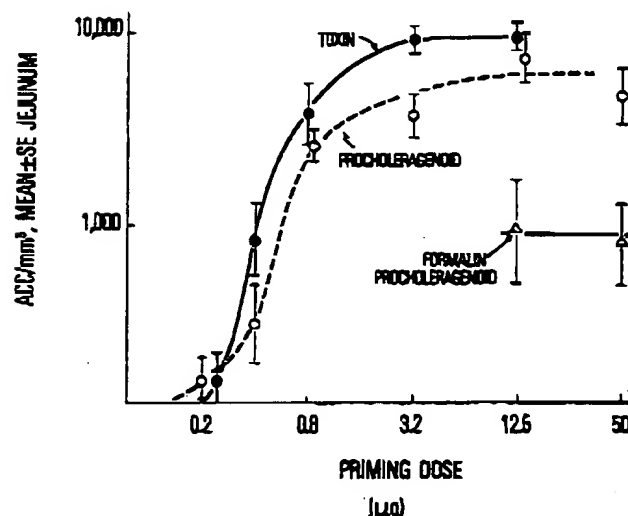


FIG. 2. Comparison of CT, procholeragenoid, and Formalin-treated procholeragenoid as primary immunogens in rat jejunum. Rats were given an intraduodenal primary immunization with CT or with one of the procholeragenoid preparations as shown. Fourteen days later, all rats were boosted intraduodenally with 12.5 μ g of CT. ACC in jejunal lamina propria were assayed 5 days later. Each point represents data from at least five rats.

incidence of mild diarrhea in dogs given only the sodium bicarbonate and Casamino Acids, with no antigen (13). In all instances, diarrhea consisted of a single soft or semiliquid stool with a volume not exceeding 25 ml. Biopsies from six immunized dogs showed vigorous jejunal ACC responses. Immunized dogs were significantly protected against diarrhea when challenged with living *V. cholerae* organisms ($P = 0.03$); they showed 83% protection against severe or lethal diarrhea and 78% protection against diarrhea of any severity. In immunized dogs, the mean stool volume passed during the first 18 h after challenge was also less, i.e., 24% of the volume observed in unimmunized controls.

DISCUSSION

Enteric immunization with CT causes vigorous mucosal IgA anti-CT responses (4, 16). Previous studies have suggested that the marked effectiveness of CT as a mucosal immunogen, especially in unprimed animals, is due to its ability to bind to GM_1 receptors present in most cell membranes and its subsequent stimulation of adenylate cyclase-mediated responses (12). Specifically, it was proposed that GM_1 binding facilitates the absorption of CT or its subsequent trapping by mucosal lymphoid tissue or both, whereas adenylate cyclase stimulation in mucosal lymphoid tissue may enhance the immune response (12). Because these features of the CT molecule also account for its enterotoxic effect, it seemed unlikely that a CT derivative would be

found that was safe, i.e., did not cause diarrhea when given orally, and yet had mucosal immunogenicity similar to that of CT. The relatively poor enteric immunogenicity of several nontoxic derivatives of CT, including cholera toxin and glutaraldehyde- and Formalin-treated toxoids, seemed to support this view (7, 12, 14, 17, 18).

The present study shows, however, that, despite its much diminished toxicity, procholeragenoid was only slightly less efficient than CT as a mucosal immunogen in rats. It was also shown that procholeragenoid was superior to cholera toxin. In that comparison, maximum ACC responses evoked by procholeragenoid equaled those caused by CT, whereas maximum responses to cholera toxin were nearly 10-fold lower. Poorer immunogenicity of cholera toxin compared with CT has been observed previously and has been attributed to its inability to enhance the immune response by stimulation of adenylate cyclase in mucosal lymphoid cells (12).

Several properties of procholeragenoid, acting separately or in concert, might account for its efficacy as a mucosal immunogen. These include its residual CT-like activity, its high molecular weight, and possibly a preserved ability to bind to GM_1 receptors on cell membranes.

The ~1% residual CT-like toxicity of procholeragenoid could not entirely explain its antigenicity. This possibility is excluded by evidence that equivalent CT doses (i.e., equal to 1% of tested procholeragenoid doses) caused much

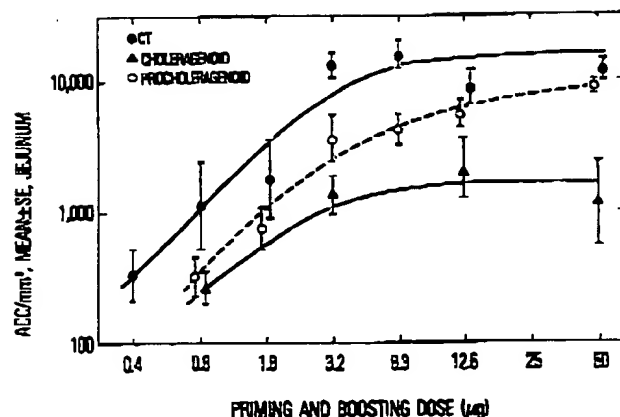


FIG. 3. Comparison of jejunal ACC responses in rats primed and boosted intraduodenally with identical doses of CT, cholera toxin, or procholera toxin. Intraduodenal priming and boosting were done with the indicated antigens and doses, using a 14-day interval. ACC in jejunal lamina propria were assayed 5 days after intraduodenal boosting. Each point represents data from at least five rats.

smaller mucosal ACC responses (Fig. 2 and 3). It is possible, however, that residual CT-like activity had an adjuvant effect upon the response to the entire procholera toxin molecule. This would be consistent with the observed poor mucosal immunogenicity of Formalin-treated procholera toxin, which was essentially non-toxic. This view is also supported by evidence that CT has an adjuvant effect in parenterally immunized mice (8).

Another possibility is that the immunogenicity of procholera toxin was partly due to its aggre-

gated state. Aggregated proteins given parenterally cause greater systemic antibody responses, due to enhanced uptake by macrophages, than do nonaggregated proteins, and the same might be true for aggregated proteins absorbed at mucosal surfaces. Aggregation might also enhance the absorption of an intestinal antigen, via M cells in the epithelium that covers mucosal lymphoid follicles (9), thus increasing the amount delivered to mucosal lymphoid tissue. The notion that aggregation contributed to the immunogenicity of procholera toxin is consistent with evidence that its high-molecular-weight components (peaks I and II) were most immunogenic, even though they did not possess more residual CT-like toxicity than the poorly immunogenic nonaggregated peak III.

Whether procholera toxin retains the capacity of CT to bind to GM₁ receptors on cell membranes has not been directly determined. This possibility was suggested, however, by evidence that preincubation of procholera toxin, or its high-molecular-weight components, with GM₁ markedly reduced their mucosal immunogenicity, as was also true for CT. These results probably reflect binding of GM₁ to exposed B subunit molecules on procholera toxin and suggest that the same interaction occurs between procholera toxin and GM₁ in cell membranes. Such binding could contribute to the effectiveness of procholera toxin as a mucosal immunogen, just as it appears to for CT (12).

This study provides the first demonstration of protective antitoxic immunity achieved by oral immunization of dogs without causing diarrhea during immunization. In previous studies, oral immunization with purified CT evoked marked protection, but also caused transient diarrhea in

TABLE 2. Relative immunogenicity of CT, procholera toxin, and separated fractions of procholera toxin

Primer dose ^a	Secondary ACC responses in rats primed with ^b :				
	CT	Procholera toxin	Peak I	Peak II	Peak III
12.5 μg	9,240 (1.2)	7,180 (1.3)	5,050 (1.4)	8,010 (1.2)	640 (1.8)
12.5 μg plus 30 μg of GM ₁	1,800 (1.5)	1,670 (1.4)	1,270 (2.3)	1,700 (1.4)	
3.2 μg	9,450 (1.2)	3,460 (1.3)	3,490 (1.3)	4,940 (1.3)	
0.8 μg	3,830 (1.4)	2,160 (1.2)	900 (1.4)	1,600 (1.8)	

^a Intraduodenal priming was with indicated dose and material. In some instances, antigen was preincubated with GM₁ ganglioside (see the text). All rats were boosted intraduodenally on day 14 with 12.5 μg of CT; ACC were enumerated in jejunal biopsies taken 5 days later.

^b Geometric mean ACC per cubic millimeter (\pm standard error); each mean contains data from at least five rats.

TABLE 3. Safety and efficacy of procholeraenoid as an oral immunogen in dogs

Dogs	Diarrhea during immunization	ACC/MM ³ , jejunum (mean \pm SE)	Serum anti-CT: proportion detectable (range, U/ml) ^a	Observation			First 18-h stool vol: mean, ml/kg of body wt (range)
				Challenge outcome (no. of dogs) ^b			
				No diarrhea	Mild diarrhea	Severe or lethal diarrhea	
Nonimmunized	2/46 (4.3%) ^c	None ^d	0/16 (≤ 2.3)	7	3	6	24 (0-92)
Immunized	4/80 (5.0%) ^c	11,000 \pm 1.2 ^f	5/16 ($\leq 2.3-7$)	14	1	1	6 (0-66)

^a Sera were obtained on the day of challenge.^b Protection of immunized dogs was statistically significant: $P = 0.03$.^c Previously reported data from fasting dogs given 50 ml of 6% NaHCO₃ followed by 100 ml of 2% Casamino Acids by mouth (13).^d Based on previously reported studies in nonimmunized dogs (15).^e Episodes of mild diarrhea observed immediately after any of the five immunizations given to 16 dogs. Considering only the first three doses of procholeraenoid, three episodes occurred after 48 immunizations (6.2% incidence).^f Biopsies were taken 6 to 7 days after the final dose of procholeraenoid ($n = 6$).

70% of animals (13); nontoxic derivatives of CT, including choleraenoid and Formalin- or glutaraldehyde-treated toxoids, did not cause diarrhea, but they also evoked little or no protection (14, 17, 18).

The vigorous immunizing regimen used in this study was designed to enhance the possibility of demonstrating protection. Whether procholeraenoid would cause protection when fewer than five doses were given, or when individual doses were smaller than 500 μ g, or both, is uncertain, but previous studies of dogs immunized orally with crude CT have shown that as few as two doses of an efficient immunogen can cause substantial protection (14).

Protection of procholeraenoid-immunized dogs was probably due entirely to antitoxin. This is likely because procholeraenoid was derived from highly purified CT which, in turn, was produced by *V. cholerae* of the opposite serotype from that used for challenge. The relative roles of antibodies to the A or B subunits of CT in this protection are, however, uncertain. Although the B subunit is the most antigenic, antibody to the A subunit has also proven highly protective in experimental animals (11). If procholeraenoid evokes a significant mucosal anti-A response, this may enhance its protective efficacy in comparison with vaccines that contain only the B subunit antigen.

The results of this study suggest that procholeraenoid would prove both safe and effective as a component of a nonliving oral cholera vaccine for humans. Such a vaccine would likely be a multiantigen product designed to stimulate enhanced (19) or synergistically protective (10, 14, 22) mucosal antibody responses, or both. Previous studies in experimental animals have shown both the importance of synergistic immu-

nity and that anti-CT and anti-lipopolysaccharide contribute substantially to it (10, 14, 22). Which other antigens of *V. cholerae* should be included, and the optimal methods for preparing and delivering such a combined vaccine, remain to be determined.

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